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The endothelial cells lining blood vessels that perfuse breast tumors present a barrier that antineoplastic agents must cross to reach the tumor cells. The purpose of this award was to provide preliminary data to support the hypothesis that tumor cells can modulate this barrier by secreting soluble factors, by affecting extracellular matrix, or by a combination of these factors. The specific aims of this project were to 1) determine tumor cell induced expression of multi-drug-resistance proteins in endothelial cells; and 2) determine tumor cell mediated changes in Adriamycin transport across endothelial cells. Experiments performed to address aim 1 revealed no tumor mediated increases in p-gp protein (a classic multi-drug resistance protein) in endothelial cells. Experiments performed to address aim 2 were hampered by the high toxicity of Adriamycin against endothelial cells. However alternative experiments were performed using transport of horseradish peroxidase and showed no tumor-mediated changes in barrier function. Although the experiments performed showed no tumor mediated changes in endothelium, these results are preliminary and the interaction of tumor and endothelial cells remains an area of research with high potential for novel chemotherapeutic strategies.

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INTRODUCTION:

The endothelial cells lining blood vessels that perfuse breast tumors present a barrier that antineoplastic agents must cross to reach the tumor cells. The purpose of this award was to provide preliminary data to support the hypothesis that tumor cells can modulate this barrier by secreting soluble factors, by affecting extracellular matrix, or by a combination of these factors. The specific aims of this project were to 1) determine tumor cell induced expression of multi-drug-resistance proteins in endothelial cells; and 2) determine tumor cell mediated changes in adriamycin transport across endothelial cells. Experiments performed to address aim 1 revealed no tumor mediated increases in p-gp protein (a classic multi-drug resistance protein) in endothelial cells. Experiments performed to address aim 2 were hampered by the high toxicity of adriamycin against endothelial cells. However alternative experiments were performed using transport of horseradish peroxidase and showed no tumor-mediated changes in barrier function. Although the experiments performed showed no tumor mediated changes in endothelium, these results are preliminary and the interaction of tumor and endothelial cells remains an area of research with high potential for novel chemotherapeutic strategies.

BODY:

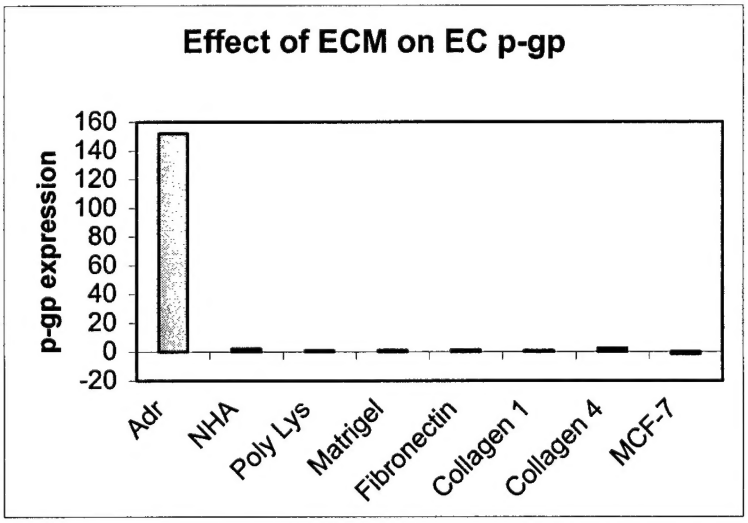
In 1971, Judah Folkman observed that tumor growth was strictly dependent on angiogenesis. Little research, however, has focused on the role of tumor endothelium as a barrier between the blood and the tumor that anticancer agents must cross to directly affect tumor cells. The focus of this project is alteration of the endothelial barrier by tumor cells as a mechanism of tumor resistance.

Endothelial cell (EC) phenotype varies widely between different tissues. EC's of the blood brain barrier (BBB) express the transporter protein, p-glycoprotein (p-gp). In vitro, non-BBB EC's cultured with astrocytes express BBB proteins. Furthermore, p-gp expression can be induced in EC's by extracellular matrix from the brain. Thus, there are factors that can alter the phenotype of EC's to express p-gp.

The initial aim of this project was to establish whether breast cancer cells could induce expression of p-gp in endothelial cells. This was determined by Western blotting of whole-cell lysates from cells treated under various conditions. Figure one shows densitometry from a representative Western blot for p-gp expression.

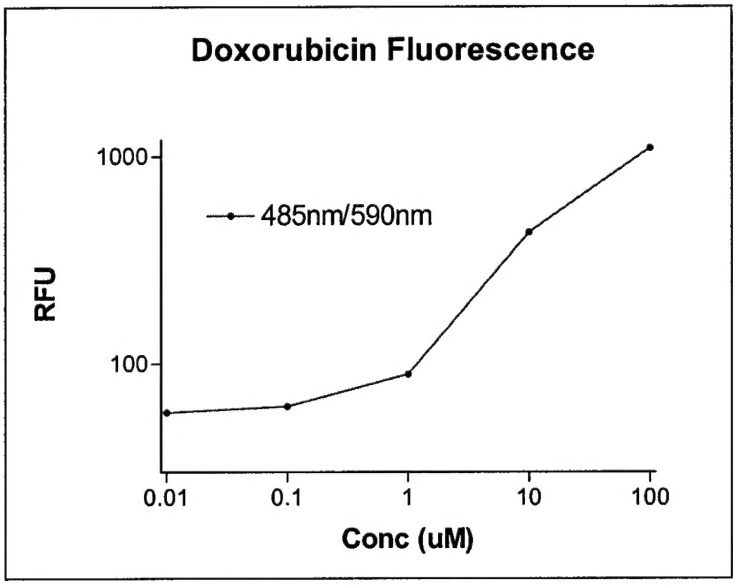
Endothelial cells were grown for 48 hours on various extracellular matrices: poly-lysine (Poly Lys) - a non-specific, negatively charged substrate; Matrigel (a trademark of BD Biosciences) - a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma; and three extracellular matrix components – fibronectin, collagen 1, and collagen 4. At the end of 48 hours, cells were scraped into PBS and equal numbers of cells were solubalized in loading buffer, electrophoresed, and then electro-blotted onto nitrocellulose membranes. The membranes were stained with ponceau S to assess total protein loading and transfer for each sample. The membranes were destained and probed for p-gp which was visualized by enhanced chemiluminescence (ECL). Expression of p-gp was quantified by densitometric analysis of the resultant film normalized to protein levels determined by the ponceau S stain. Along with the endothelial cells, two breast cancer cell lines from the National Cancer Institute screen were included as positive and negative controls: NCI/ADR-RES cells (Adr) constitutively express p-gp, and MCF-7 cells which do not express p-gp. Also assayed were normal human astrocytes (NHA)

Figure 1



The goal of Aim 2 was to evaluate adriamycin uptake and transport by endothelial cells. We performed initial experiments to determine the limits of detection for adriamycin based on fluorescence. Varying concentrations of adriamycin in solution were assayed in a fluorometer at an excitation wavelength of 425nm. Fluorescence was measured as a ratio of the emmision at 590nm divided by 485nm. The lower limit of detection was on the order of 0.1 to 1 uM adriamycin (Figure 2).

Figure 2

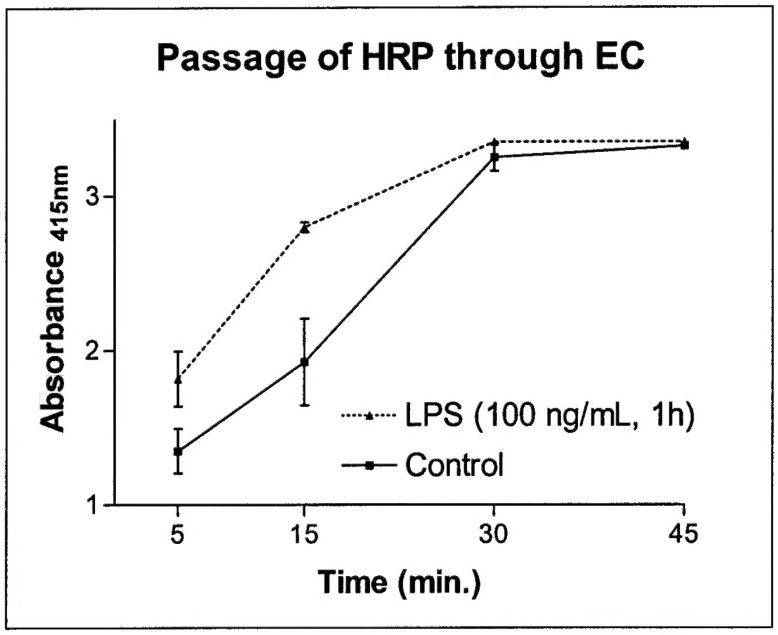


Unfortunately, experiments to determine basal uptake and transport of adriamycin across endothelial cell layers were hampered by unexpectedly high toxicity of adriamycin to the endothelial cells. Cytotoxicity testing using an MTT-based assay revealed an IC_{50} between 0.01 uM and 0.1 uM following 24-hour exposure of endothelial cell to adriamycin.

We therefore began to develop alternative experiments using an assay based on the passage of horseradish peroxidase (HRP) through the endothelial cell monolayer. Briefly, endothelial cells were plated in Transwell tissue clusters (Corning) and incubated for 24 hours. Following treatment, HRP was added to the apical reservoir. At various timepoints, samples were taken from the basal reservoir and assayed for HRP activity using a colorimetric assay.

Preliminary results using LPS to stimulate disruption of the endothelial layer indicate that this technique may be a suitable alternative for evaluating the transport of compounds (such as anticancer drugs) across an endothelial layer (Figure 3). Unfortunately, this assay failed to produce consistent results within the timeframe of the project.

Figure 3



KEY RESEARCH ACCOMPLISHMENTS:
None

REPORTABLE OUTCOMES:
None

CONCLUSIONS:

These experiments indicate no changes in p-glycoprotein expression in human umbilical vein endothelial cells (HUVEC) grown on various extracellular matrix proteins including a tumor cell matrix (matrigel). Other experiments (not shown) indicated that growing endothelial cells in medium conditioned by breast cancer cells or human astrocytes also failed to induce expression of p-glycoprotein in HUVEC. Finally, co-culture of HUVEC with tumor cells failed to produce detectable p-glycoprotein in cellular lysates from the co-cultured cells. We concluded, therefore, that p-glycoprotein expression was not significantly upregulated *in vitro* by soluble or insoluble factors. It is possible that p-glycoprotein expression in endothelial cells is differently regulated *in vivo*. Certainly, the scientific literature is filled with instances of failed attempts to induce expression of proteins *in vitro* that are normally expressed by cells *in vivo*.

Although our experiments were not able to evaluate the effect of tumor cells extracellular matrix or soluble factors on HUVEC permeability, these results are preliminary and further protocol optimization may yield reportable outcomes.

Finally, the interactions of tumor cells and endothelial cells remain a highly interesting area of research. This award, while not producing the positive results desired, provided valuable training for the principle investigator as well as producing guidance for further investigations.

REFERENCES:

Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. 1971 Nov 18;285(21):1182-6

Hurwitz AA, Berman JW, Rashbaum WK, Lyman WD. Human fetal astrocytes induce the expression of blood-brain barrier specific proteins by autologous endothelial cells. Brain Res. 1993 Oct 22;625(2):238-43.

Tatsuta T, Naito M, Mikami K, Tsuruo T. Enhanced expression by the brain matrix of P-glycoprotein in brain capillary endothelial cells. Cell Growth Differ. 1994 Oct;5(10):1145-52.

Schlaifer D, Laurent G, Chittal S, Tsuruo T, Soues S, et al. Immunohistochemical detection of multidrug resistance associated P-glycoprotein in tumour and stromal cells of human cancers. Br J Cancer. 1990 Aug;62(2):177-82.

Iwahana M, Utoguchi N, Mayumi T, Goryo M, Okada K. Drug resistance and P-glycoprotein expression in endothelial cells of newly formed capillaries induced by tumors. Anticancer Res. 1998 Jul-Aug;18(4C):2977-80.